



## Evaluation of a wide range of pepper genotypes for regeneration and transformation with an *Agrobacterium tumefaciens* shooter strain

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### Abstract

A regeneration protocol developed for the *Agrobacterium tumefaciens*-mediated transformation of pepper (*Capsicum annuum* L.) was used to evaluate the potential for genetic transformation of 107 doubled haploid (DH) pepper genotypes belonging to 12 main cultivar groups. The genotypes were scored on the basis of the ratio of regenerated shoots compared to the commercial cultivar Fehérözön, which exhibited 30–70% regeneration from the total number of explants. Fifty DH genotypes responded, representing all the of the main cultivar groups, 31 of which showed regeneration frequencies similar to or better than cv. Fehérözön. By using a shooter strain with a binary vector harbouring the cucumber mosaic virus (CMV) coat protein (CP) gene and selecting regenerants on media containing kanamycin, the transformability of the pepper genotypes as well as resistance to CMV were simultaneously analyzed. The regenerated plants were selected and tested by PCR to detect the CP gene. Eighteen PCR-positive DH plants and six PCR-positive commercial Fehérözön cultivar plants were regenerated and rooted by grafting. In the case of three transgenic genotypes, presence of the CMV CP was confirmed by PCR in the T1 generation indicating the stable integration of the CP gene. In the progeny of one DH genotype of the Demre type, the presence of the transgene as well as resistance to the virus were demonstrated in the T2 generation.

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### 1. Introduction

A number of viruses are reported to infect pepper (*Capsicum annuum* L.), amongst which cucumber mosaic virus (CMV) is one of the most important, causing decreases in yield and quality (Hull, 2002). The most effective and environmentally friendly way to control virus diseases is to develop resistant varieties, which can be achieved faster via genetic engineering than by traditional breeding.

Genetic engineering for virus resistance using the coat protein (CP)-mediated approach was first reported by Roger Beachy and co-workers in 1986 (Powell-Abel et al., 1986). Since then, it has become a routinely used method for obtaining

virus-resistant crops (Beachy, 1990, 1997), such as transgenic papaya hybrids resistant to papaya ringspot virus in Hawaii (Gonsalves, 1998), potato genotypes resistant to a broad spectrum of viruses (Tacke et al., 1996), and grape and squash genotypes resistant to relevant virus diseases (Fuchs and Gonsalves, 2007).

Several species are still recalcitrant to genetic modification and regeneration, while in other species the transformation efficiency is highly dependent on the genotype. Even if a specific cultivar is transformable, the yield of this procedure is often very low. Pepper is among the species where very few successful transformations have been reported. Zhu et al. (1996) reported a relatively high regeneration rate from various explants of a Chinese pepper cultivar after *Agrobacterium*-mediated transformation with the CMV CP gene, but no convincing molecular evidence was presented for stable transformation, nor were the

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transformants tested for virus resistance. Shin et al. (2002) reported the successful transformation of the pepper variety cv. Golden Tower, carrying the L1 resistance gene, with the cucumber mosaic virus and Tomato mosaic virus CP genes. In this study, the hypersensitive reaction was utilized as a convenient and reliable transgenic selection marker. The authors obtained seven transgenic pepper genotypes harbouring both CP genes.

An efficient regeneration system is a critical requirement if major varieties of pepper are to be improved through the production of transgenic plants. During the study presented in this paper, the responsiveness of 107 pepper genotypes were tested in the ‘shooter’ *Agrobacterium* transformation system developed by Mihálka et al. (2003). For each variety group several DH genotypes were used.

## 2. Materials and methods

### 2.1. Construction of the transformation vector

The coat protein gene of the Ns-CMV RNA 3 clone, belonging to subgroup 1 of cucumoviruses (Divéki et al., 2004) was amplified by PCR using a primer complementary to the 3′ end of RNA 3 (5′-AGATCTGGTACCCCTAGGGG-3′) and a primer corresponding to the nt 1170–1187 segment of the intergenic region (5′-GGGAGCTCTCCGCGAGATTGCGTT-3′, the incorporated *Sac*I site is underlined). The PCR product was digested with *Sac*I restriction endonuclease, and ligated into a pUC19 plasmid previously cut with *Sac*I and *Sma*I. From this clone the CP gene was subcloned into pBluescript II KS+ with the *Eco*RI and *Xba*I restriction endonucleases, resulting in the clone pKS+NscP. The CP gene from pKS+NscP was excised with *Sac*I restriction endonuclease and ligated into *Sac*I-digested pRGNeo (Mihálka et al., 2000), resulting in the pRGNeoNsCP transformation vector (Fig. 1).

### 2.2. Bacterial strains

The ‘shooter’ *Agrobacterium* strain (‘ShooterG’) used throughout the experiments was previously characterized by Mihálka et al. (2003) as a mutant strain inducing extensive shoot formation and minimal tumor formation in tobacco leaf disk transformation. The pRGNeoNsCP binary vector was introduced into ShooterG by triparental mating (Ditta et al., 1980) using the pRK2013 helper vector (Figurski and Helsinki, 1979). The resulting strain, ShooterG-NsCP, was grown in YEB (Vervliet et al., 1975) or in minimal AB (Chilton et al., 1974) medium supplemented with the appropriate antibiotics (100 mg/l kanamycin, 100 mg/l rifampicin).

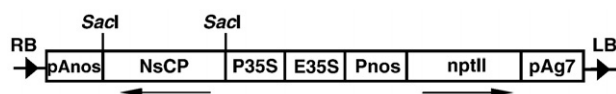


Fig. 1. Schematic diagram of the pRGNeoNsCP binary vector used in the transformation experiments.

### 2.3. Production of doubled haploid plants

Doubled haploid (DH) pepper plants were produced via an anther culture protocol based on the work of Dumas de Vaulx et al. (1981) and Mitykó et al. (1995).

### 2.4. Plant material and co-culturing of explants with *Agrobacterium*

In order to produce explant material, seeds of 107 doubled haploid (DH) pepper genotypes belonging to 12 cultivar groups (listed in Table 1), and of cv. Fehérözön as a control variety were germinated on MS medium under dim light. Each DH genotype was numbered and blind-tested against the standard cultivar Fehérözön, which proved to be the most responsive variety in earlier work. Tips from the cotyledons of 16 to 21-day-old aseptic seedlings were cut (Fig. 2), and these cotyledon explants were co-cultivated with *Agrobacterium* as described by Mihálka et al. (2003). For each DH genotype 50 to 100 cotyledons were transformed. After 2 days of co-cultivation, the explants were transferred to fresh liquid MSB5gl medium [MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 20 g/l glucose] supplemented with 500 mg/l cefotaxime and incubated for at least 4–6 h to eliminate *Agrobacterium*.

### 2.5. Tissue culture and plant regeneration

All tissue culture and plant regeneration steps followed in this study were previously described by Mihálka et al. (2003), and a short version of the whole transformation protocol was recently summarized (Mihálka and Balázs, 2006). During the regeneration phase neither hormones nor kanamycin were added to the medium. Kanamycin (150 mg/l) selection was carried out only during the 4th and 5th passage of excised regenerants on rooting medium (MS basal medium supplemented with 0.5 mg/l IAA, 15 g/l glucose, 15 g/l maltose), after the elimination of *Agrobacterium*.

### 2.6. Rooting and grafting techniques

After 3 weeks of selection, plants were removed from the rooting medium and grafted. Stocks were derived from six-week-old (four to five leaf stage) rooted seedlings, preferably belonging to the same variety as the scions. The stems of the scion and stock were cut at right angles, with 2–3 leaves remaining on the scion. Then the stem of the scion was cut in a wedge with a scalpel and the tapered end was fitted into a cleft cut in the end of the stock. The graft was held firmly with a silicon rubber clip (BATO Trading B.V., The Netherlands) as described by Tóth et al. (2007). Sterile grafts were grown on rooting medium (described above) until the first new leaves appeared (about 10–18 days). Then the grafts were transferred into Jiffy-7 pellets (Jiffy International AS, [www.jiffypot.com](http://www.jiffypot.com)) and maintained in a growth chamber under 16 h light (warm white light: F29 Tungsram tubes and L37 Osram Fluora tubes) at 24 °C. After two weeks the plants were transferred to the greenhouse.

Table 1  
Regeneration responses and transformation results of doubled haploid genotypes in the transformation system compared to those optimized for the commercial Fehérözön cultivar

Cultivar group (type)	No. of DH genotypes tested	No. of DH genotypes exhibiting shoot regeneration	Score of response <sup>a</sup> (No. of DH genotypes)			<i>In vitro</i> regenerated and rooted PCR-positive plants	No. of individual T0 plants tested for resistance
			Weak	Medium	Strong		
Cecei <sup>b</sup>	17	9	4	2	3	1	0
Fehérözön	4	3	0	1	2	3	0
Almapaprika	1	1	1	0	0	0	0
Dolma	7	3	1	2	0	0	0
Blocky	13	1	1	0	0	0	0
Lamuyo	24	11	3	5	3	7	3
Corno	3	2	1	0	1	1	1
Kapia	5	4	2	1	1	0	0
Rapidus	2	2	0	0	2	0	0
Carliston	7	5	1	1	3	3	2
Demre	6	4	2	0	2	3	1
Hungarian spice pepper (paprika)	18	5	3	2	0	0	0
Total	107	50	19	14	17	18	7

To obtain at least 50 cotyledons for each transformation experiment 50–100 seeds were germinated from 10 DH genotypes. The number of explants used for each genotype varied based on the germination capability of the different genotypes.

<sup>a</sup> Weak, medium, strong: produced fewer, the same number or more shoots than the commercial cultivar Fehérözön.

<sup>b</sup> Characteristics of the plant materials: Cecei — traditional Hungarian type, conical in shape, ivory to red in color, with a delicious sweet taste; Fehérözön — fruit type similar to Cecei, but the growth habit is determinate (fastigiate); Almapaprika — traditional Hungarian type, smooth, apple-shaped fruit, whitish yellow to red, hot; Dolma — traditional Turkish type, small, blocky shape, very thin fruit flesh, pale green to red, sweet; Blocky — California Wonder or Bell type, dark green, red or yellow, sweet; Lamuyo — elongated blocky type, dark green, red or yellow, sweet; Corno — traditional Italian and Spanish type, long, horn-shaped, dark green, red or yellow, sweet; Kapia — traditional Bulgarian and Turkish type, long, smooth, dark green or red, sweet; Rapidus — Hungarian, elongated, yellow waxy or red (banana type), hot; Carliston — Turkish, elongated, pale green or red, sweet; Demre — traditional Turkish type, shape similar to Cayenne, green or red, mild or hot; Hungarian spice pepper (paprika) — determinate (fastigiate) or normal growth habit, long, dark green or red, hot or sweet, consumed as ground powder.

## 2.7. Analysis of transgenic plants

To test for the presence of the CMV CP transgene, genomic DNA was isolated from *in vitro* regenerated (T0) or greenhouse-grown T1 or T2 plants as described by Dellaporta et al. (1983). The polymerase chain reaction was carried out under standard conditions with 30 s denaturation, 30 s annealing and 45 s extension at 94 °C, 60 °C and 72 °C respectively. The specific primers (NsCP-for: 5' CATGGACAAATCTGAATCAAC-CAGTGC 3' and NsCP-rev: 5' CGGAATCAGACTGGGAGCA-

CTCC 3') amplified a 660 nt sequence of the introduced coat protein gene.

Virus resistance was tested in the T1 generation. Seeds of T0 plants were planted separately in individual glass test tubes containing MS medium plus 150 mg/l kanamycin for germination. Germinating kanamycin-resistant plants were transferred once or twice, as antibiotics break down rapidly in the medium.

Virus resistance was tested by mechanically inoculating young greenhouse-grown seedlings (2–3 true leaves) with 20 µg/ml purified virion using a sterile glass spatula and celite powder. To check whether infection had been established, total RNA was extracted 3–4 weeks after inoculation (RNeasy Plant Mini Kit, Qiagen) in addition to visual screening. First strand cDNA synthesis from the viral RNA was initiated by the CMV1-rev oligonucleotide (5' ATCATTGTAGCACCTCATAGCA 3'). For the reverse transcription, RevertAid reverse transcriptase was used, applying the protocol provided by the manufacturer (Fermentas, Lithuania). The subsequent PCR employed the primers CMV1-for (5' GGAGGTTATGATATGGGCTTT 3') and CMV1-rev to amplify a 582 bp fragment of CMV RNA1. Thirty PCR cycles were run with 30 s at 94 °C, 30 s at 52 °C and 45 s at 72 °C.

## 3. Results and discussion

Due to varying consumer demands in different countries and to the different cultivation methods used, many genotypes of pepper are grown. Based on long experience (Mihálka et al., 2000, 2003)



Fig. 2. 16–21-day-old pepper seedlings suitable for transformation. Tip-cut cotyledons were used as explants.



there are extreme differences in regeneration capacity among the cultivated genotypes. The pepper transformation protocols published by various authors have focused on a single genotype (Zhu et al., 1996; Kim et al., 1997; Manoharan et al., 1998), or in one case on four genotypes (Li et al., 2003), so the strong genotype dependency of the applied regeneration system cannot be excluded. To establish a large-scale transformation and regeneration system for pepper it was obvious that the most important cultivated genotypes should be involved in the study. Following successful experiments previously carried out on the cultivar Fehérözön (Mihálka et al., 2000, 2003), the regeneration experiments were extended using the ‘ShooterG’ mutant *Agrobacterium* strain to include doubled haploids from almost all the important groups of cultivars. The use of doubled haploid genotypes ensured the genetic uniformity of the plant material used in these experiments, which is an enormous advantage for further breeding applications. Additionally, the binary vector carried a transgene providing an important trait for pepper, CMV resistance. A total of 107 DH genotypes from 12 cultivar groups were selected and used in the experiments. The characteristics of the experimental plant material are described in the footnote to Table 1.

In the preliminary experiments the germination periods differed and depended on the given genotypes. Cotyledons are suitable for regeneration for up to four days, after which they are no longer capable of regeneration. At this stage the cotyledons are diverging, but are not yet flat (see Fig. 2). The selection process should not start with the simultaneous elimination of the transforming bacteria and selection of transgenic tissue as both drugs tend to cause yellowing, making it difficult to differentiate between the effect of selection and the toxic effect of dead bacteria.

Compared to the control (cv. Fehérözön), regeneration following transformation using the ShooterG strain was weaker for 19 DH genotypes, similar in 14 genotypes and stronger in 17 genotypes (Table 1). Regeneration also depended on the quality of the cotyledon tissue.

A binary vector, containing the CMV CP gene and the neomycin phosphotransferase gene (*nptII*) as a selection marker was used for transformation (Fig. 1). Kanamycin-resistant regenerated shoots were tested for the CMV CP gene as described above. PCR screening for CMV-CP showed that a total of 18 regenerants, belonging to 6 cultivar groups (Cecei,



Fig. 4. CMV-susceptible (left) and resistant (right) phenotype of the T2 progeny of the DH17 Demre line two months after inoculation with CMV Ns strain. Stunting, leaf deformation, and vein clearing caused by CMV-Ns can be clearly detected on the susceptible plant, while the resistant one is symptomless.

Fehérözön, Lamuyo, Corno, Charleston, Demre: Table 1), were positive for the CP gene, indicating transgene integration. PCR was conducted both on *in vitro* and adult plants from the greenhouse. The enhanced activity of the *ipt* gene in the ShooterG strain caused problems in the rooting ability of the regenerants. The “rooting” medium described in Materials and methods ensured better elongation and a reduction in the amount of callus tissue, but only resulted in rooting in approx. 10% of cases. Thus, shoots testing positive for PCR-CP were grown by grafting under sterile conditions, as described above. Although 100% of the grafts took, not all the plants could be grown to the seed production stage. Some plants died during the greenhouse phase, and plants were lost even after repeated grafting of shoots of the same transgenic genotype, which might be the result of the enhanced activity of the *ipt* gene. The acclimatisation period varied, but was mostly about 5 weeks, whereafter the plants were too old to be challenged by the virus.

Therefore, virus inoculation tests were carried out on T1 plants. For selection, the seeds had to be planted in separate tubes, because kanamycin-sensitive seeds produce phenolic compounds when they necrotise, which would affect the resistant seeds. As the

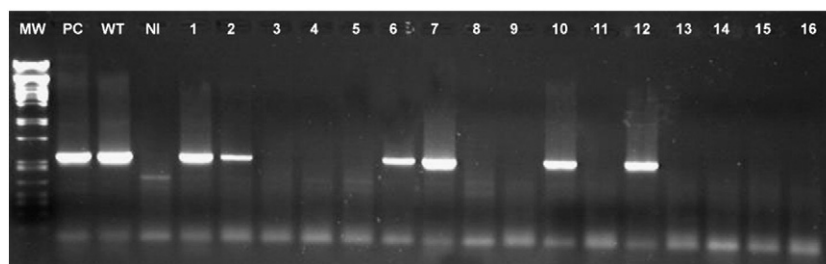


Fig. 3. Confirmation of the presence or absence of CMV-Ns replication in inoculated transgenic T2 pepper progeny of the Demre genotype DH17 by RT-PCR. Results of sixteen out of 26 inoculated plants are shown. In plants #1, 2, 6, 7, 10 and 12 a 582-bp CMV-specific band is detectable, while in plants #3, 4, 5, 8, 9, 11, 13, 14, 15 and 16 the presence of the virus could not be verified, which was in accordance with the healthy phenotype observed. MW:  $\lambda$ PstI molecular weight marker, PC: plasmid vector harbouring the cloned RNA1 of CMV-Ns, WT: non-transgenic control inoculated with CMV-Ns, NI: non-inoculated control.

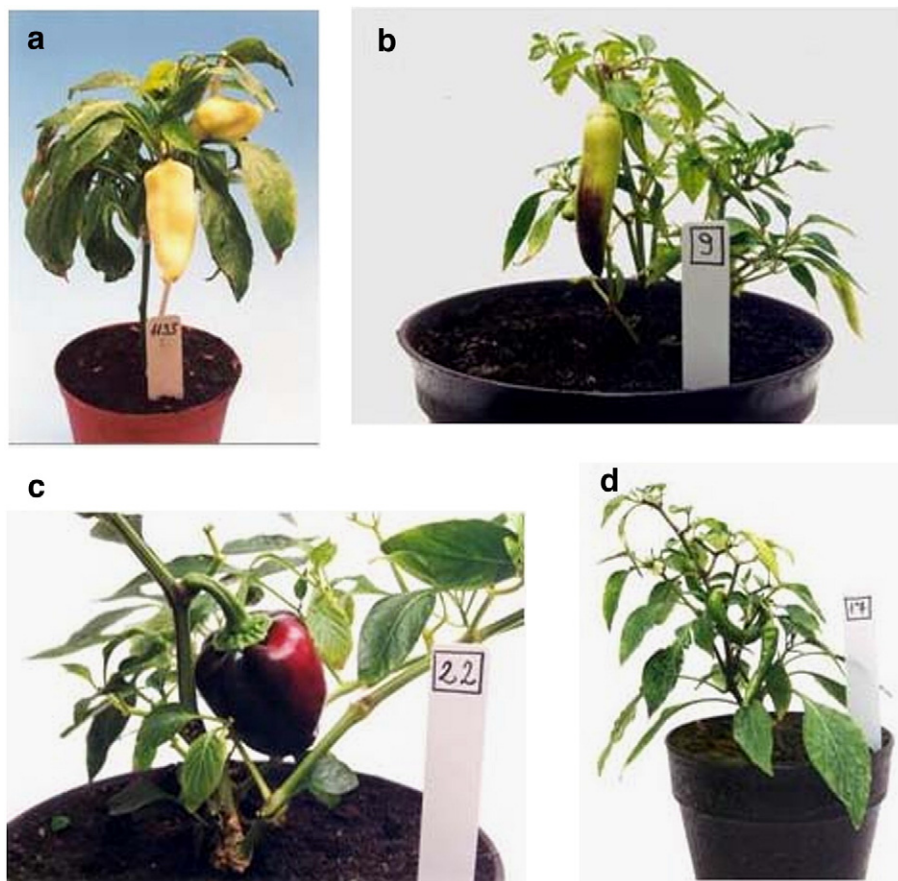


Fig. 5. Greenhouse-grown transgenic T0 pepper plants with fruit. a. Fehérözön b. DH9 (Carliston type) c. DH22 (Lamuyo type) d. DH17 (Demre type).

germination process was prolonged (the selection process took two to six weeks) all the virus resistance tests had to be performed individually. Eleven putative T0 transgenic genotypes representing six DH genotypes (7 transgenic genotypes) and 4 control Fehérözön transgenic plants were examined. From the twenty seeds of each transgenic line planted, a total of seven kanamycin-resistant shoots were obtained from two Fehérözön and one Demre genotype (DH17). These T1 plants were tested by PCR and found to be CP-positive, indicating transgene integration. The other PCR-positive T0 plants, which were tested several times but did not produce kanamycin-resistant seeds, presumably did not have a stably integrated transgene. The transgenic DH17 genotype was further analyzed in the T2 generation, where 32 kanamycin-resistant T2 plants were obtained, thirty-one of which harboured the CMV coat protein gene. Twenty-six of these plants were inoculated with CMV as described above. Seventeen plants proved to have varying levels of resistance, while susceptibility due to the absence of the gene was confirmed by RT-PCR (Fig. 3) and by the presence of CMV symptoms (leaf rugosity, stunting and vein yellowing; Fig. 4) in nine plants. Fig. 5 shows the fruits of various greenhouse-grown T0 transgenic pepper genotypes.

Using this shooter mutant-based transformation system, efficient regeneration can be achieved on hormone-free medium in a wide range of important pepper genotypes. Out of the 107 DH genotypes tested, 50 responded, 31 of which – belonging to

ten different cultivar groups – exhibited similar or better regeneration capacity compared to cv. Fehérözön. Three transgenic pepper plants were obtained. In one of those an agronomically important trait, cucumber mosaic virus tolerance, was also tested and verified.

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